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The pathologic findings os postmortem lymph nodes (LN) from supraclavicular, axillary, mediastinal, mesenteric, and inguinal sites were compared in 5 Western blot confirmed HIV-infected asymptomatic intravenous drug addicts (IVDA) and 8 HIV seronegative control IVDA. The predominant LN pattern was classified as follicular hyperplasia (FH), sinus histiocytosis (SH), and lymphoid depletion (LD). The percent distribution of these histologic changes in HIV-positive (and HIV-negative) individuals was 64% (47%) FH, 20% (31%) LD, and 15% (22%) SH. Plasmacytosis was prominent in 15 of 25 (60%) LN from HIV seropositive individuals, but absent in controls. Frozen and paraffin immunohistochemistry of HIV+ LN with FH demonstrated p24 antigen within germinal centers in a distribution similar to CD35+ endritic reticulum cells, but not in controls. HIV-1 RNA could not be detected by in situ hybridization in LN which were HIV-DNA positive by polymerase chain reaction, probably because of postmortem RNA degradation, but was demonstrated in all HIV-positive surgically-acquired control tissues. LN changes in early HIV infection are nonspecific. HIV infection in LN can only be diagnosed by the demonstration of HIV genome, antigens or viral particles.

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Principal Investigator's Signature Date

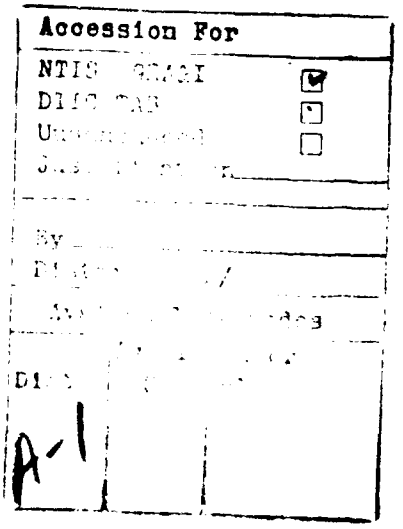
INTRODUCTION

Nature of Problem. The long-term goal of this work is to establish a laboratory capable of detecting HIV-1 in tissue samples. Cellular localization as well as (semi) quantitation are parts of this goal. These techniques may be useful in staging disease and monitoring therapy. The tissues studied in this proposal will be exclusively from patients with early (i.e., non-symptomatic) HIV disease and will exclude AIDS patents.

Background of previous work. There have been several studies that have identified HIV-1 in tissues by several techniques. Immunohistochemistry and *in situ* hybridization have been shown to be successful in demonstrating HIV RNA in lymph nodes and central nervous system tissues (1-16,19). The polymerase chain reaction (PCR) has also demonstrated HIV-1 in autopsy as well as surgical tissues from patients with HIV disease (17-18). Most of these studies have been performed on tissues from patients with symptomatic HIV disease.

Purpose of present work. The initial goals of this grant project are to collect autopsy tissues from HIV-positive asymptomatic drug addicts who die unexpectedly from drug overdoses. A wide sampling of autopsy tissues will be subjected to various techniques in identifying and localizing HIV-1. The viral burden, cellular sites of replication and progression of viral disease will be studied.

Methods of approach. Three technologies will be employed: immunohistochemistry, *in situ* hybridization, and polymerase chain reaction. The first two techniques demonstrate viral antigens and nucleic acids, respectively, and provide information on cellular localization. PCR provides a sensitive technique for viral DNA detection and can be modified for semi-quantification of viral burden.



BODY

EXPERIMENTAL METHODS

Tissue procurement. Between the months of January and April, 1990, tissues were obtained from autopsies performed at the Maryland Medical Examiner's Office in Baltimore, Maryland, and the District of Columbia Medical Examiner's Office with the collaboration of Dr. John Smialek (Baltimore) and Dr. Matthias Okoye. Tissue collection at Washington D.C. was terminated because of difficulties that arose at that office. At the time of autopsy, deaths due to drug overdose were brought to our attention, and a screening ELISA for the presence of serum antibodies to HIV-1 was performed. Lymphoid, central nervous system, myocardial, and genitourinary tissues were harvested and fixed in buffered formalin and quick-frozen and stored at -80C. Tissues were harvested in both seropositive and seronegative individuals. The results of screening ELISA were confirmed at the state laboratory by Western blot methods.

Tissue controls Three types of positive tissues controls wjere used. 1. Infected H9 cell pellets acquired from SRA laboratories, Rockville, were prepared at different dilutions and frozen into cell blocks that were sectioned in a similar manner as frozen tissues or fixed and processed. 2. Human T-cell lymphoma tissues that were grown in nude mice were recieved courtesy of Dr. Neal Wetherall, Vanderbilt University (20); some had been infected in vivo with HIV-1 and viral replication demonstrated at Vanderbilt. 3. Frozen lymph nodes from a prostitute with HIV-1 related lymphadenopathy were obtained from the D.C. Medical Examiner's Office and positivity for HIV-1 antigens and nucleic acids established. 4. Placental tissues from seropositive and seronegative placentas were obtained courtesy of Dr. Adolfo Firpo (AIDS registry, AFIP). Negative controls consisted of seronegative drug addict tissues collected during the course of the study.

Histologic evaluation. The pathologic evaluation of the lymphoid tissues was undertaken in collaboration with Dr. G. Frizzera, Chairman of Hematic and Lymph Node Pathology, AFIP. The morphologic alterations in lymph nodes of seropositive as well as seronegative individuals was studied with his cooperation. The pathologic evaluation of central nervous system tissues was undertaken by Dr. Jorge Ribas, who has special expertise in this area.

Immunohistochemical techniques. Immunohistochemical methods followed standard procedures using the avidin-biotin technique (Vectastain ABC kit). All slides were presilanated for tissue adhesion. When necessary, tissue sections were predigested in 0.05% Sigma VIII protease for three minutes at 37 degrees C. Positive and negative controls were run in parallel for every stain. Negative controls were run with each tissues, and consisted of a similar technique with the replacement of the specific primary antibody with normal serum of the same species,

or omission of this step. Positive controls for lymphoid markers lymph node tissue obtained from seronegative controls. Positive controls for HIV antigens included mouse tissues, frozen lymph nodes obtained from a patient known to have ARC; this autopsy performed at the D.C. Medical Examiner's office.

Antibodies. Commercially available antibodies were utilized, both monoclonal and polyclonal, against lymphoid markers as well as HIV-1 viral antigens. Enzo Diagnostics is the supplier for MA 935, a macrophage antibody used at a dilution of 1:50; Dako is the supplier for anti-B cell marker L26, used at a dilution of 1:100, CD3 (pan-T) used at a dilution of 1:100, UCHL-1 (pan-T) used at a dilution of 1:200, lysozyme (dilution a:100), and anti HIV-1 p24, used at a dilution of 1:5; Becton Dickinson is the supplier for anti CD4 and CD8, both used at a dilution of 1:20; Accu-specs is the supplier for sheep polyclonal anti HIV-1 p24 antigen, used at a dilution of 1:10; DuPont is the supplier for anti HIV-1 p24, used at a dilution of 1:100, anti-HIV-1 gp41, used at a dilution of 1:100. In addition, polyclonal antisera raised in rabbits against whole inactivated HIV-1 virus are being prepared in collaboration with WRAIR retrovirology.

In situ hybridization techniques Frozen sections are immediately fixed in 3% paraformaldehyde for 5 minutes after allowing to dry and come to RT and can then be stored in 70% ethanol at -4C indefinitely. They are then rinsed in 2XSSC for 10 minutes before beginning step 6. Alternatively, frozen sections can be stored indefinitely at -70C; this method is not preferred as it does not inactivate virus. If paraffin sections are used, the first five steps are needed.

1. Take section to water, through xylene and graded alcohol.
2. Rinse in dH_2O and two changes1X PBS.
3. Treat section with 10mg/ml protease K solution in 0.1M Tris HCl, pH 8.0 + 50 mM EDTA for 10 minutes at 37C. NOTE: Length of incubation varies with tissue type and degree of fixation.
4. Rinse slides in DEPC-treated water for 2 minutes
5. Rinse slides in 0.1M triethanolamine pH 8.0; blot dry on filter paper.
6. Add 500 ul acetic anhydride to dry staining dish. Add 200 ml 0.1M TEA (final concentration 0.25%v/v). Mix solution and add slide rack with slides to the dish. Incubate at room temperature of 10 minutes.
7. Rinse in 2X sodium chloride and Sodium citrate (SSC solution).
8. Dehydrate in graded alcohols (70% 1 min, 80% 1 min, 90% 2 min, 100% 2 min).
9. Add 73 ul of 1:1 dybridization solution and formamide, 25 ul probe (500,000 cpm) 2 ul 5M dithiothreitol in 1% SDS for total 100ul.
10. Overlay probe onto sections, and cover with parafilm. Incubate at 50C overnight in a humid chamber.
11. Remove the probe solution (cover slips/parafilm should come off), and rinse in 2 changes of 2X SSC for 5 minutes each.

12. Make 1 l 0.5M NaCl, 10mM Tris pH 8, 1 mM EDTA (100 ml 5M NaCl, 10 ml 1M Tris HCl pH 8, 2 ml 0.5 EDTA, dH_2O to 1 liter). Add 20 ug/ml RNAse A. Treat for 30 minutes at room temperature. Rinse in RNAse buffer 30 additional minutes. For DNA target only, perform this step before probe incubation (step 12; of course DNA probes must be used in this case.
13. Wash in 2X SSC at 50C for 1 hour.
14. Wash in 0.2 SSC at 60C for 2 hours.
15. Dehydrate in graded ethanols containing 300 mM ammonium acetate; air dry.
16. Perform autoradiography according to standard techniques. Negative controls (labelled sense and irrelevant, usually beta-actin probes) were run with each run, as well as positive control tissues. Specificity of reaction was also determined by pre-RNase digestion of positive control tissues, which negated the reaction in every case.

Probes. RNA ^{35}S labelled probes for in situ hybridization specific for HIV-1 were purchased from Oncor, Gaithersburg MD, and Lofstrand Labs, Rockville MD. The Oncor probe is 1-2 kb in length and spans 10-20% length of HIV genome, including the 3' LTR and nef regions. The Lofstrand probe is a cocktail of three probes (gag/pol 1246-3410 site, sor 4200-5300, and env 6700-7700).

Polymerase chain reaction technique. To extraction DNA from paraffin blocks, a single 5-10 micron section is cut from the block and placed in a 500 ul Eppendorf tube. 400 ul of xylene is added for deparaffinization, and centrifuged for 5 minutes. The xylene is decanted, 400 ul 95% ethanol added, and decanted after recentrifugation. Acetone is added for desiccation. After drying, the tissue pellets are incubated overnight at 37C in 50 to 100 ul of extraction solution (100 mmol/L Tris hydrochloride, 4 mmol/L ethylenediaminetetraacetate, 400 mg/L proteinase K, pH8.0). The samples are then boiled for 7 minutes to inactivate proteinase K. The samples are centrifuged, and 0.5 to 10 ul of the extraction solutions containing DNA are amplified by PCR.

To extract DNA from frozen sections, two 6-micron sections of fresh-frozen tissue are cut from the block and paced in in an Eppendorf tube. A fresh microtome blade is used for every specimen to avoid carryover of DNA from one specimen to the next. The embedding medium is dissolved by vortexing the sections in 800 ul distilled water. After addition of 400 ul ethanol, the tube is vortexed again and centrifuged at 14,000 rpm for five minutes. The supernatant is decanted and the sample resuspended by vortexing in 800 ul ethanol. After another five minute centrifugation, the supernatant is decanted and any remaining ethanol is removed with a microcapillary pipet.

Cycling parameters were 1.5 minutes at 94C (denaturation), 1.5 minutes at 42C (elongation) and 3 minutes at 72C

(reannealing). Reagents and kits were purchased from Perkin Elmer Cetus. To avoid contamination, positive pressure pipets were used. Tissue samples were cut with disposable blades to prevent cross-over. With each run, two negative controls were run (sterile water and negative tissue control). Positive controls included primers for human B-IFN as well as a positive tissue control. Core buffer was prepared according to kit instructions, and reaction was overlain with 4 drops mineral oil before cycling. Amplified product was detected by electrophoresis (agarose 1.5% Nu-Sieve, 3.5% Seakem), with ethidium bromide, and confirmed with P-32 labelled probes by liquid hybridization.

Primers and probes. Primers used for polymerase chain reaction detection of HIV-1 were the SK 29 and SK30 primers which have been shown to be sensitive and specific for HIV-1. Their respective sequences are ACT AGG GAA CCC ACT GCT and GGT CTG AGG GAT CTC TA. The probe used to identify amplified product was the SK31 sequence: ACC AGA GTC ACA CAA CAG ACG GGC ACA CAC TAC T. To determine that amplifiable DNA was extracted, an internal primer pair for human interferon is used:B-IFN GAG CCG AGC TCG CTT AAT CTC CTC AGG G (28 MER)/ GCA ACC TTT CGA AGC CTT TGC TCT GGC (27 mer).
These probes were synthesized at the AFIP by the infectious disease department, with the kind assistant of Dr. T. Hadfield.

RESULTS

Patient demographics. Patient data are given in table 1. The majority of patients were black males aged 25-40.
Histopathologic findings (Table 2). There was no difference in the histologic findings in lymph node between seropositive and seronegative individuals, with the exception of a greater incidence in plasmacytosis in seropositive individuals. There was a range of findings including the non-specific reactive changes of sinus histiocytosis, follicular hyperplasia, and paracortical T-cell stimulation. Specific alterations of ARC-related lymphadenopathy were not observed, although one lymph node in a seropositive individual demonstrated irregular outlines of reactive follicles, a finding that has been seen in HIV-related lymphadenopathy as well as other conditions. Histologic findings of AIDS-related changes in brain tissues (syncytial cell formation, foci of necrosis with lymphocytic infiltrates) were not observed in sections of brain. Myocarditis was not observed in any of the heart tissues examined. Sections of other organs were unremarkable.
Immunohistochemical findings. Results of lymphoid markers supported results of histopathologic study. Reactive follicles, when present, demonstrated positivity with B-cell antibodies as well as CD35 (anti-interdigitating reticulum cell antibodies), as expected. Paracortical areas stains with pan-T cell markers UCH1

and CD3. There was no difference in pattern or degree of staining with T-cell subsets, ie CD 4 and CD8 in lymph nodes from seroreactive vs. seronegative individuals. This determination was made subjectively without the aid of morphologic quantitative techniques. p24 antigen staining was reproducibly found only using the antibody from DAKO; the reagents from DuPont and Accu Sys were repeatedly attempted with variable results. Antigen positivity was demonstrated only in lymph nodes from seropositive individuals. The localization of antigen in every case was limited to reactive follicles. The pattern of staining was similar to that of CD35, eg reticulum interdigitating cells. However, not every reactive follicle stained, and not every lymph node in a given individual was positive. Tabulation of positivity is currently under way and complete results will be forthcoming in the next several weeks.
In situ hybridization results. Every tissue was hybridized with two labelled positive probes, from Oncor and Lofstrand laboratories, as well as sense and irrelevant probes. In each run, positive controls were strongly positive, and these included cell controls and xerotransplanted infected cell lines. The probe from Oncor resulted in high background and non-specific staining in test tissues. Non-specific staining involved eosinophils and pigment-containing macrophages. The Lofstrand probe demonstrated cleaner and more easily interpretable data. Although placental tissues from seropositive patients, also used as positive controls, demonstrated scattered positive cells in maternal cells in a distribution consistent with expected results, autopsy tissues were invariably negative for consistently positive cells.

PCR results. Bands consistent with the length of the HIV-1 amplification product were readily identified from frozen tissues (Table 3). Most of these were lymphoid tissues. The specificity of these bands was demonstrated by liquid hybridization using a specific ^{32}-P labelled probe. Amplification of the B-IFN internal control sequence was accomplished in all cases, and negative lanes were clean in both distilled water and negative tissue lanes. Amplification of paraffin-embedded tissue is under way, and preliminary results indicate that sensitivity is greatly lessened by formalin fixation. As the results in Table 3 indicate, some tissues from sero-negative patients are positive. There results are being investigated, and may represent contamination, or HIV-1 infection in seronegative high-risk individuals. The results of critical data are being run in parallel with the HIV-1 PCR laboratories of SRA, Rockville.

CONCLUSIONS

Lymph node morphology in HIV-1 seropositive asymptomatic drug addicts and seronegative controls is non-specific. Plasmacytosis is more prominent in the seropositive group. Immunohistochemistry demonstrates HIV-1 antigens in a significant proportion of lymph nodes within reactive germinal centers in a distribution similar to interdigitating reticulum cells. We were unable to demonstrate HIV-1 RNA within these lymph nodes, perhaps because of post-mortem autolysis and RNA lability. By PCR, HIV-1 can easily be demonstrated in frozen lymph nodes from these patients, but the sensitivity of formalin fixed tissues is far less. Virus is less often demonstrated in cardiac and genitourinary tissues by PCR.

Table 1. Patient tissue sources

Number	age	sex	race	ELISA	Western blot
188	31	f	b	-	
271	34	m	b	+	+
329	51	m	b	+	+
343	34	m	b	+	+
993	35	f	c	-	
462	30	m	b	-	
504	32	f	b	+	+
512	22	m	b	-	
549	44	m	b	-	
689	37	m	b	-	
810	40	m	c	-	
816	36	m	b	-	
897	40	m	b	+	+
926	32	m	b	+	+

Table 2A. Lymph node histopathology, seropositive cases

case	axillary	supraclavicular	mediastinal	mesenteric	inguinal
271		FH, PC	PC, FH	PC, LD	FH, PC
329	FH, PC	FH, PC	PC, SH, LD	PC, LD	PC, LD
504	FH, PC	PC, LD	PC, FH, PCH	FH	PC, FH
897	FH, SH	SH, LD	FH	FH, SH	FH, PCH, SH
926	FH, PCH, SH	PC, SH, LD	PC, PCH, SH	FH, SH, PCH	FH, irreg

Table 2B. Lymph node histopathology, seronegative cases

case	axillary	supraclavicular	mediastinal	mesenteric	inguinal
188	SH, LD	SH, LD	LD, SH		
993			SH, LD	SH, LD	
462	SH, LD	LD	LD	SH, LD	
512	FH, SH	PC, FH, SH		FH, SH	FH, PCH, SH
549	FH, SH		FH, SH		
689	SH, LD	LD, SH	SH, LD	SH, LD	LD, SH
810	FH, PCH	PCH	FH, SH		FH, PCH, SH
816	SH, LD		LD, SH		SH, LD

Abbreviations: SH, sinus histiocytosis; LD, lymphoid depletion; FH, follicular hyperplasia; PCH paracortical hyperplasia; irreg irregular; PC plasmacytosis

Table 3. PCR results

Case sero-reactivity	tissue (frozen) tons	ax	scl	med	mes HIV B-IFN	PP EtBr/LH	ing	spl	kid	pros	brain	heart
926	+/+	+/+	+/+	+/+	+/+		+/+	-/+			-/-	-/+
+	+	+	+	+	+		+	+			+	+
329	+/+		+/+	+/+	+/+	-/+	-/-	+/+	-/-	+/+	-/-	
+	+		+	+	+	+	+	+	+	+	+	
504	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	-/-		+/+	+/+
+	+	+	+	+	+	+	+	+	+		+	+
462	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/+	-/-	-/-	-/-	
-	+	+	+	+	+	+	+	+	+	+	+	
993				-/-	-/-	+/+		-/-	-/-		-/-	
-				+	+	+		+	+		+	
512	-/-		-/-		-/+	-/-	-/-	-/-	-/-	-/-	-/-	-/+
-												
810	-/+	-/+	-/-	-/-		-/-	-/-	-/-	-/-	-/-	-/-	-/-
-	+	+	+	+		+	+	+	+	+	+	+
816		-/-		-/-		-/-		-/-	-/-	-/-		
		+		+		+		+	+	+		
-												

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